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Identification of specific antigenic epitope at N-terminal segment of enterovirus 71 (EV-71) VP1 protein and characterization of its use in recombinant form for early diagnosis of EV-71 infection

Jianhua Zhang[a,b], **Bingfu Jiang**[a], **Mingjie Xu**[a], **Xing Dai**[c], **Michael A. Purdy**[d], and **Jihong Meng**[a,*]

[a]Department of Microbiology and Immunology, School of Medicine, Southeast University, Nanjing, Jiangsu, PR China

[b]Department of Microbiology and Immunology, School of Medicine, Shaoxing University, Shaoxing, Zhejiang, PR China

[c]Department of Dermatology, Affiliated Zhongda Hospital, School of Medicine, Southeast University, Nanjing, Jiangsu, PR China

[d]Division of Viral Hepatitis, Centers for Disease Control and Prevention, Atlanta, GA, USA

Abstract

Human enterovirus 71 (EV-71) is the main etiologic agent of hand, foot and mouth disease (HFMD). We sought to identify EV-71 specific antigens and develop serologic assays for acute-phase EV-71 infection. A series of truncated proteins within the N-terminal 100 amino acids (aa) of EV-71 VP1 was expressed in *Escherichia coli*. Western blot (WB) analysis showed that positions around 11–21 aa contain EV-71-specific antigenic sites, whereas positions 1–5 and 51–100 contain epitopes shared with human coxsackievirus A16 (CV-A16) and human echovirus 6 (E-6). The N-terminal truncated protein of VP1, $VP1_{6–43}$, exhibited good stability and was recognized by anti-EV-71 specific rabbit sera. Alignment analysis showed that $VP1_{6–43}$ is highly conserved among EV-71 strains from different genotypes but was heterologous among other enteroviruses. When the GST-$VP1_{6–43}$ fusion protein was incorporated as antibody-capture agent in a WB assay and an ELISA for detecting anti-EV-71 IgM in human sera, sensitivities of 91.7% and 77.8% were achieved, respectively, with 100% specificity for both. The characterized EV-71 VP1 protein truncated to positions 6–43 aa has potential as an antigen for detection of anti-EV-71 IgM for early diagnosis of EV-71 infection in a WB format.

Keywords

Enterovirus 71; Hand, Foot and mouth disease; Viral capsid protein; Early diagnosis

*Corresponding author. Tel.: +86 25 8327 2386. jihongmeng@163.com (J. Meng)..

1. Introduction

Human enterovirus 71 (EV-71) and human coxsackievirus A16 (CV-A16), belonging to human enterovirus species A of the genus *Enterovirus* within the family *Picornaviridae*, are the two major causative agents of hand, foot and mouth disease (HFMD). HFMD is a common febrile disease, which mainly affects infants and young children, resulting in the appearance of vesicular rashes on hands, feet, and buccal mucosa (Hagiwara et al., 1978). In severe cases, EV-71 infection may lead to neurological complications, such as aseptic meningitis, brainstem encephalitis and acute flaccid paralysis with high incidence of fatality (Lee et al., 2009). EV-71, first identified in California in 1969 (Schmidt et al., 1974), has spread to cause widespread epidemics around the world, especially in Southeast Asia (AbuBakar et al., 1999; Ahmad, 2000; Chan et al., 2000; Chang, 2008; Liu et al., 2000). In China, EV-71 infection has resulted in at least 1600 deaths from 2008 to 2012 (Xing et al., 2014). EV-71 is now regarded as the most important neurotropic enterovirus in the Asia-Pacific region in the post-polio eradication era (Bible et al., 2007; Wong et al., 2010). As there are no effective antiviral agents or vaccines available for treatment and prevention of EV-71 infection, early and rapid diagnosis of EV-71 infection is critical to implementing timely clinical management and public health intervention.

The standard laboratory diagnosis of EV-71 infection is based on a conventional virus isolation and identification by neutralization test or immunofluorescence assay, which is labor intensive, subjective, and time-consuming. Molecular diagnostic assays for EV-71 based on conventional or real-time RT-PCR have been developed and widely used in clinical practice, which require dedicated, expensive equipment and maintenance of separate areas to avoid contamination. So, these methods are unsuitable as point of care assays in most community clinics in developing countries. Serological diagnosis using enzyme immunoassay (EIA) is an alternative approach owing to its usage of basic laboratory equipment. In recent years, a few IgM-capture ELISA assays have been developed for early and rapid diagnosis of EV-71 infection (Tano et al., 2002; Tsao et al., 2002; Wang et al., 2004; Xu et al., 2010). These approaches use, as antibody-capture agent, purified whole EV-71 virion, which can bind to antibodies that cross-react with other enteroviruses.

VP1 is a major and immunodominant capsid protein of EV-71. Recombinant VP1 protein has been investigated as an antigen for serological diagnosis (Shih et al., 2000). In a previous study we have found that full-length VP1 possesses strong cross-reactivity with sera raised against CV-A16 and human echovirus 6 (E-6) (Zhang et al., 2012). We also found that the major immunoreactive region of the VP1 protein is located at the N-terminus, between amino acid (aa) positions 1–100. We report here that recombinant truncated protein VP1 located between positions 6–43 aa is a potential antigen for use in the early serologic diagnosis of EV-71 infection.

2. Materials and methods

2.1. Cells and viruses

Human rhabdomyosarcoma (RD) cells were grown in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum plus 100 IU of penicillin and 100 μg/ml of streptomycin. Three local enterovirus strains, designated EV-71/MAS01/AH/CHN/09 (GenBank accession No. JQ409495), CV-A16/MAS01/AH/CHN/10 (GenBank accession No. JQ409499) and E-6/NJ01/JS/CHN/10 (GenBank accession No. JQ409486), were isolated and cultured in RD cells.

2.2. Serum specimens

As described previously, three antisera against EV-71, CV-A16 and E-6 were prepared by immunizing New Zealand white rabbits with the three heat-inactivated viruses respectively (Zhang et al., 2012). Serum samples were obtained from 51 children diagnosed with HFMD upon admission to the Nanjing Children's Hospital. Virus-specific diagnosis was established by the detection of RNA from EV-71, CV-A16 or other enteroviruses in throat or anal swabs following reverse-transcriptase-PCR with primers specific for EV-71, CV-A16 and pan-enterovirus (PEV) (Liu et al., 2011). Negative control sera were collected from 87 healthy children (<5 years old) in kindergartens in Shaoxing city, who were confirmed to be uninfected with EV-71, having tested negative serologically for antibodies against EV-71 using an *in vitro* neutralization assay as described previously (Zhang et al., 2012).

2.3. Construction of the recombinant plasmids

The VP1 gene derived from strain EV-71/MAS01/AH/CHN/09 was used as template to amplify the coding regions of the N-terminal VP1 using the Expand High Fidelity PCR system (Roche Diagnostics GmbH, Mannheim, Germany). All forward primers were designed with the *EcoR* I restriction site, and the reverse primers were designed with the *Not* I restriction site and a stop codon at the 5′ end (Table 1). The cloned fragments were digested with *EcoR* I and *Not* I (NEB, Ipswich, MA, USA), purified and inserted into the prokaryotic expression vector pGEX-5X-1 (GE Healthcare, Piscataway, NJ, USA) to generate recombinant plasmids. Successful ligation was verified by sequencing.

2.4. Expression and purification of the recombinant proteins

Escherichia coli BL21 (DE3) transformed with the recombinant plasmids was cultured in Luria broth (LB) medium supplemented with ampicillin (50 μg/ml) at 37 °C. When the cultures reached an optical density of 0.6–0.8 at 600 nm, the cells were induced with isopropyl-β-D-thiogalactopyranoside (IPTG; 1 mM) and growth was continued for 3–5 h. The cells were harvested by centrifuging at 8000 × *g* for 10 min at 4 °C. The pellets were suspended in Tris–HCl buffer (50 mM; pH 7.9) supplemented with Triton X-100 (0.1%; v/v), lysozyme (1 mg/ml) and $MgCl_2$ (4 mM). Subsequently, the lysates were treated with DNase (5 mg/ml) and RNase (5 mg/ml) for 30 min at 25 °C. After centrifugation at 20,000 × *g* for 15 min at 4 °C, the clear supernatant was collected and used for solubility analysis and purification. All the GST fusion proteins were purified using glutathione sepharose 4B (GE Healthcare, Piscataway, NJ, USA) according to the GST gene fusion system handbook. The

protein purity and concentration were measured using the SDS-PAGE and the Bradford assay.

2.5. Western blot (WB) analysis

Approximately 1 μg of the GST fusion protein was run in 15% SDS-PAGE under denaturing conditions, and transferred to nitrocellulose membrane. The blotted membrane was blocked with TBST containing 5% (m/v) non-fat milk. Serum samples were diluted 1:100 in blocking buffer, added to the membranes and incubated overnight at 4 °C. The membrane was rinsed and incubated for 1 h with affinity-purified goat anti-rabbit IgG, or goat anti-human IgM conjugated to horseradish peroxidase (KPL, Gaithersburg, MD, USA) diluted 1:2000 in blocking buffer. After washing, color development was carried out with 3,3′ -diaminobenzidine. To test all human serum specimens simultaneously, WB analysis was performed using a 20-channel Mini-Protean II multiscreen apparatus (Bio-Rad, Hercules, CA, USA).

2.6. Indirect ELISA

Purified GST fusion proteins, diluted to a final working concentration (10 ng/μl) in PBS (pH 7.4) were added to microwell immunoassay strips to a final volume of 100 μl per well. The strips were coated overnight at 4 °C and blocked with 1% BSA in PBS. After washing, 100 μl of the serum samples at a dilution of 1:100 were added to each well, and the strips were incubated for 45 min at 37 °C. After washing again, HRP-conjugated goat antihuman IgM (KPL, Gaithersburg, MD, USA) was added at a dilution of 1:5000. Finally, the reaction was developed using 100 μl/well of tetramethylbenzidine (TMB) (Sigma–Aldrich, St Louis, MO, USA) substrate, and optical density values were determined.

2.7. Computational and sequence analyses

The amino acid sequence of the truncated VP1 protein fragment was used as query sequence for BLAST analysis (blastp 2.2.26+) against all enterovirus entries in the National Center of Biotechnology Information (NCBI) database. Amino acid sequence alignment was performed using the ClustalW algorithm of DNAStar MegAlign.

3. Results

3.1. Expression and purification of truncated VP1 proteins

Nine overlapping PCR fragments encoding different N terminal segments of VP1 within positions 1–100 aa were amplified separately and inserted into pGEX-5X-1 (Fig. 1a). Expression of cloned transformants was induced with protein was in agreement with the expected molecular weight (Table 2). All fusion proteins were soluble, and purified using glutathione sepharose 4B under native conditions. The purity of all the purified proteins was high by analysis with SDS-PAGE (Fig. 1b). The fusion proteins $VP1_{1–100}$, $VP1_{6–100}$, $VP1_{11–100}$ and $VP1_{21–100}$ were unstable (Fig. 1b, lanes 1–4). The smaller proteins $VP_{1–50}$ and $VP_{6–50}$ were also unstable (Fig. 1b, lanes 5 and 8). However, after removing seven or more amino acid residues from the carboxyl terminus of $VP_{1–50}$ or $VP_{6–50}$, fusion proteins $VP1_{1–40}$, $VP1_{1–30}$ and $VP1_{6–43}$ were stable (Fig. 1b, lanes 6, 7 and 9). These observations suggested that positions 44–50 aa confer instability in these proteins.

3.2. Mapping of EV-71 specific antigenic fragment by WB analysis

Immunoreactivity of the nine truncated VP1 proteins was tested using WB assay with rabbit antisera against EV-71, CV-A16 and E-6, respectively. $VP1_{1–100}$ and its C-terminal truncated proteins, $VP1_{1–50}$, $VP1_{1–40}$ and $VP1_{1–30}$, reacted strongly with anti-EV-71 sera. Two N-terminal truncated proteins ($VP1_{6–100}$ and $VP1_{11–100}$) also showed strong reactivity to anti-EV-71. However, after truncation of 10 amino acids from $VP1_{11–100}$ the reaction of $VP1_{21–100}$ to anti-EV-71 became weaker. These results suggested that the VP1 region around positions 11–21 aa contains a major immunoreactive site to anti-EV-71 (Table 2). As demonstrated previously (Zhang et al., 2012) and again in this study, $VP1_{1–100}$ exhibited cross-reactivity with rabbit antisera against CV-A16 and E-6. Three C-terminal truncated proteins, $VP1_{1–50}$, $VP1_{1–40}$ and $VP1_{1–30}$, also reacted with anti-CV-A16 sera, but did not react with anti-E-6 sera. Three N-terminal truncated proteins, $VP1_{6–100}$, $VP1_{11–100}$ and $VP1_{21–100}$ reacted with both anti-E-6 and anti-CV-A16 sera. However, the cross-reactivity to anti-CV-A16 was reduced comparing to the reactivity of $VP1_{1–100}$. The remaining two proteins, $VP1_{6–50}$ and $VP1_{6–43}$, were reactive to anti-EV-71 sera only, and unreactive to anti-CV-A16 or anti-E-6 sera (Table 2). These results suggest that EV-71's VP1 region between positions 51–100 aa contains epitopes shared with E-6 and CV-A16. There is an additional epitope shared with CV-A16 at the N-terminus, between positions 1–5 aa. Taking into account the instability of $VP1_{6–50}$, protein $VP1_{6–43}$ was chosen for further analysis as a candidate for EV-71-specific diagnostic reagent.

3.3. Alignment analysis of aa sequences of EV-71 $VP1_{6–43}$

Protein-protein BLAST analysis revealed that the amino acid sequence of EV-71 $VP1_{6–43}$ contains high homology (89.5–100%) among heterologous EV-71 strains from different sub-genotypes, and relatively low homology (26–55%) with other viruses from species A enteroviruses. No significant homology was found among enteroviruses from species B, C or D except for poliovirus (homology 23–29%) (Table 3). Alignment using the ClustalW algorithm of the program MegAlign (DNAStar) also showed that the sequence at positions 6–43 aa of VP1 was highly conserved among all EV-71 strains from the different genotypes and sub-genotypes and different from the sequences of strains of CV-A16 and E-6 (Fig. 2).

3.4. Detection of anti-EV-71 IgM in human sera using the fusion protein GST-$VP1_{6–43}$

Human serum specimens were collected from 51 HFMD patients infected with EV-71 (n = 36), CV-A16 (n = 8) and other enteroviruses or non-enteroviruses (n = 7). Negative control sera were collected from 87 healthy children. The WB assay using recombinant protein GST-$VP1_{6–43}$ showed that sera from 33 of the 36 EV-71-infected patients were positive (91.7%), and the remaining 15 sera from HFMD patients infected with non EV-71 were negative (Table 4). The utility of GST-$VP1_{6–43}$ was also investigated using indirect IgM ELISA. Checkerboard titration revealed that the most appropriate dilutions for the fusion protein and HRP-conjugated goat antihuman IgM were 1:200 (1 μg/ml) and 1:5000 respectively. After the 87 negative control sera were tested, the cut-off OD value was calculated as the mean plus two standard deviations of the negative sample. Mean OD value was 0.074 and the standard deviation was 0.053; the cut-off value was set as 0.180. Of the 36 sera collected from EV-71 infected patients, 28 sera (77.8%) were positive, and all 15

sera from patients with non EV-71 infections were negative (Table 4). These data shown that none of false positive results was observed in both ELISA and immuno-blot assay, but the sensitivity of anti-EV-71 IgM detection depends on the method used (WB or ELISA) although the same antigen GST-$VP1_{6–43}$ is applied.

4. Discussion

Early diagnosis is essential for effective clinical management and control of infectious diseases. Generally, IgM antibody will be detectable very early after primary infection for EV-71 infection. Therefore, detection of anti-EV-71 IgM based on enzyme immunoassay (EIA) should be an ideal method for early and rapid diagnosis of EV-71 infections. The key to developing this technique is to choose an appropriate antigen with high sensitivity and specificity.

Recent studies have reported the purification of whole EV-71 particles used as antigen to develop μ-capture ELISA for anti-EV-71 IgM detection (Tano et al., 2002; Tsao et al., 2002; Wang et al., 2004).Xu et al. (2010) assessed the performance of anti-EV-71 IgM detection using μ-capture ELISA, which showed an acceptable sensitivity in EV-71 infected patients, and a satisfactory specificity in non-HFMD children and healthy adults. However, out of 332 sera from cases of non-EV-71 enterovirus infection, 38 sera (11.4%) showed anti-EV-71 IgM positivity. This substantial false negative rate was assumed to be due to cross reactivity to common epitopes among enteroviruses. In addition, the preparation of whole EV-71 particles was cumbersome and time-consuming because of the use of cell culture followed by virus purification and inactivation, and the need to monitor the inactivation process.

The EV-71 capsid protein VP1 had also been considered for use as a diagnostic antigen. Recombinant EV-71 VP1 expressed in *E. coli* can react specifically with antibodies from patients infected with EV-71 by the WB assay (Shih et al., 2000). However, in our previous study EV-71 VP1 possessed strong cross-reactivity with antisera against CV-A16 and E-6, which suggested recombinant EV-71 VP1 is not a suitable antigen for specific serodiagnosis of EV-71 infection (Zhang et al., 2012). Further study showed that the major immunoreactive region of the VP1 protein was located in the N-terminal part at position 1–100 aa. However, truncated His-$VP1_{1–100}$ protein still contains cross-reactive sites recognized by antibodies against CV-A16 and E-6 (Zhang et al., 2012).

In this study, GST-tag fusion protein $VP1_{1–100}$ and a series of truncated proteins were expressed in *E. coli* and investigated for their immunoreactivity. WB analyses showed that $VP1_{1–100}$ and its C-terminal truncated proteins ($VP1_{1–50}$, $VP1_{1–40}$ and $VP1_{1–30}$) reacted strongly with antibodies against EV-71. Two N-terminal truncated proteins ($VP1_{6–100}$, $VP1_{11–100}$) also showed strong reactivity. However, $VP1_{21–100}$, truncated 20 aa from N-terminus, reacted weakly. Recently, two non-neutralizing linear epitopes were found at positions 3–8 and 12–19 aa of VP1 (Lim et al., 2013; Man-Li et al., 2012). The truncated proteins ($VP1_{6–100}$, $VP1_{11–100}$) do not contain a complete copy of the 3–8 epitope. Compared with $VP1_{1–100}$, however, the immunoreactivity of $VP1_{6–100}$ and $VP1_{11–100}$ was not significantly reduced. These results suggest that a VP1 N-terminal region at positions

around 11–21 aa contains the major EV-71 immunoreactive sites. Similar with our results, a linear epitope located at 6–20 aa of EV-71 VP1 was identified recently by a non-neutralizing monoclonal antibody (MAb 27) and highly conserved among all EV-71 subgenotypes (Xu et al., 2013).

The N-terminal VP1 region spanning from positions 51 to 100 aa contains CV-A16 and E-6 cross-reactive sites, and the first 5 aa in the N-terminus contains an additional CV-A16-cross-reactive site. Without these cross-reactive sites, the $VP1_{6–50}$ and $VP1_{6–43}$ should specifically recognize antibodies against EV-71. Due to the instability of the $VP1_{6–50}$, only the $VP1_{6–43}$ was chosen for further study for its potential application in early diagnosis of EV-71 infection.

Sequence analysis showed that the $VP1_{6–43}$ was highly conserved among all strains that represent genotypes A, B and C of EV-71, and exhibited relatively low homology with other enteroviruses in species A–D. As further proof of concept, WB analysis and an indirect ELISA based on the GST-$VP1_{6–43}$ were developed to detect anti-EV-71 IgM antibodies. The sensitivity of the WB assay and the ELISA was 91.7% (33/36) and 77.8% (28/36), respectively, and the specificity was 100% for the both tests. The relatively lower sensitivity of the indirect ELISA may be attributed to anti-EV-71-specific IgG in test sera, which competed with EV-71-specific IgM for antigen binding, thereby generating false-negative results. Foo et al. (2008) reported a very similar result, in which a GST fusion protein harboring an EV-71-specific antigenic epitope did not react with anti-EV-71 IgG antibodies in ELISA but was strongly reactive to the antibodies in WB assay. Therefore, the indirect ELISA format, perhaps, is not a suitable approach for anti-EV-71 antibody detection with some special antigens.

In summary, we reported here that a truncated protein segment located at N-terminus (6–43 aa) of EV-71 VP1 contains an EV-71-specific antigenic site. It can be easily prepared with high solubility, purity and stability from recombinant *E. coli*. Although the present sensitivity is not enough to make a diagnostic kit with the antigen for anti-EV-71 IgM detection, its high specificity will encourage us to do further studies to improve the assays. This novel assay may be developed as immunoblot strip format. Moreover, changing the indirect format to IgM-capture format may also be an effective way to raise the sensitivity. Making multiple epitope or mosaic epitope antigen based on the findings in the present or other studies may also need to be considered. Efforts to explore more EV-71-specific epitopes and prepare recombinant antigens containing such epitopes are essential for establishing a simple and economic serodiagnostic assay for early diagnosis of EV-71 infection.

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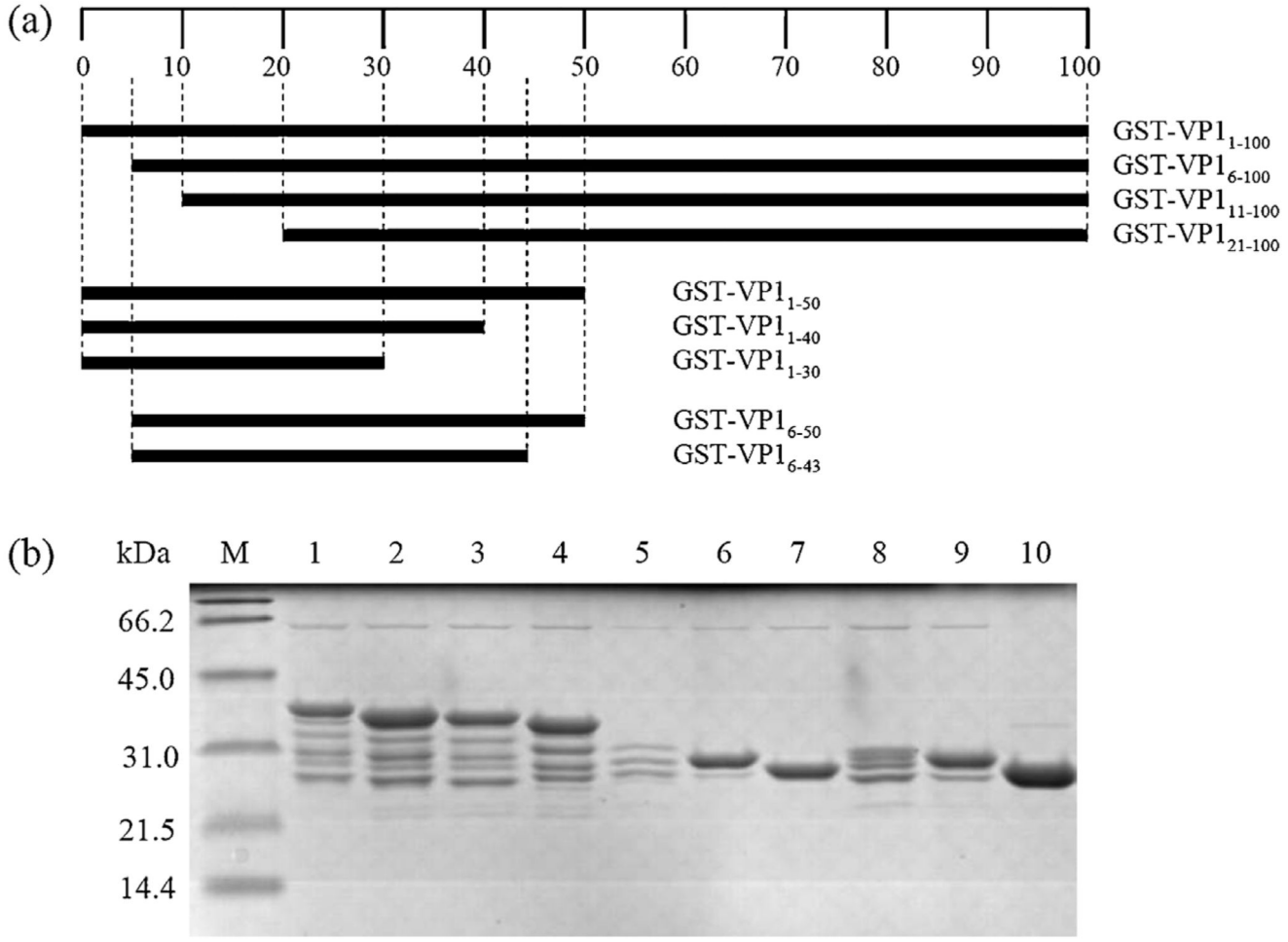

Fig. 1.

Expression and purification of EV-71 VP1 truncated proteins located along the first 100 aa of the N-terminus. (a) Schematic representation of truncated proteins expressed in *E. coli*. (b) Purified recombinant proteins were separated on a 15% SDS-PAGE gel and stained with Coomassie blue. Lane M: protein molecular weight marker in kDa; lane 1, VP11-100; lane 2–4: $VP1_{6–100}$, $VP1_{11–100}$, and $VP1_{21–100}$; lane 5–7: $VP1_{1–50}$, $VP1_{1–40}$, and $VP1_{1–30}$; lane 8, $VP1_{6–50}$; lane 9, $VP1_{6–43}$; lane 10: GST.

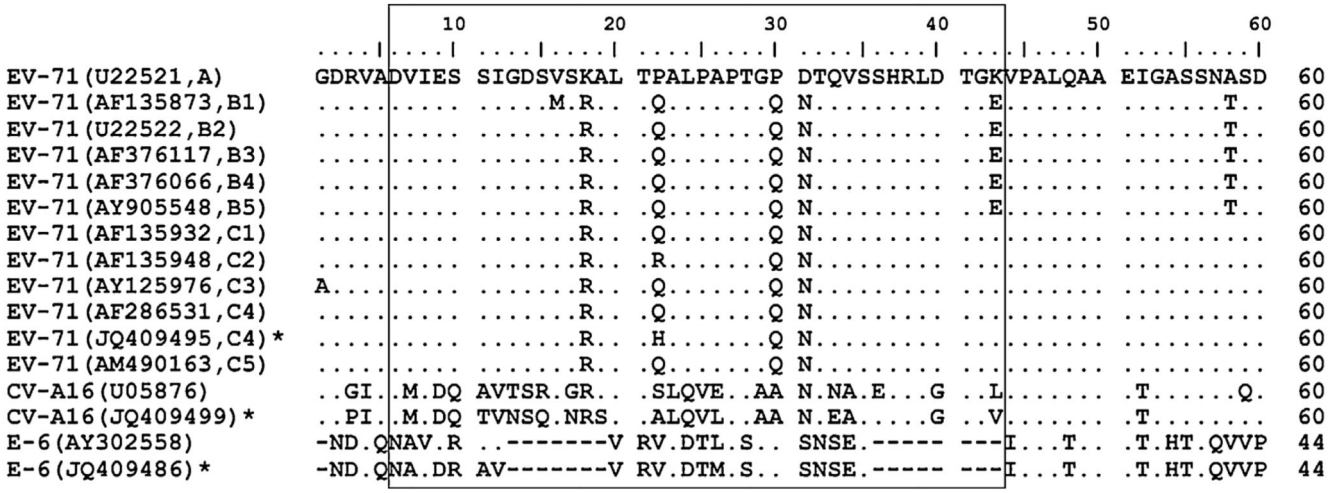

Fig. 2.

Alignment of EV-71 VP1 sequences at positions 1–50 aa with heterologous EV-71 strains from different sub-genotypes, CV-A16 and E-6 strains. The VP1 region 6–43 aa is boxed. Asterisks (*) denote three local enterovirus strains.

Table 1

Oligonucleotides primers for the construction of recombinant plasmids.

Primers[a]	Sequence (5′ → 3′)[b]
VP1-F1 (EcoR I)	CCG***GAATTC***GGAGATAGGGTGGCAGATGT
VP1-F6 (EcoR I)	CCG***GAATTC***GATGTAATTGAAAGTTCCATAGG
VP1-F11 (EcoR I)	CCG***GAATTC***TCTATAGGAGATAGCGTGAG
VP1-F21 (EcoR I)	CCG***GAATTC***ACTCACGCTCTACCAGCACC
VP1-R30 (Not I)	TAT***GCGGCCGC***CTACTGGCCTGTGGGTGCTGGT
VP1-R40 (Not I)	TAT***GCGGCCGC***CTAATCCAGTCGATGACTGCT
VP1-R43 (Not I)	TAT***GCGGCCGC***CTACTTGCCTGTATCCAGTCGATG
VP1-R50 (Not I)	TAT***GCGGCCGC***CTAAGCAGCTTGGAGTGCTGAAACC
VP1-R100 (Not I)	TAT***GCGGCCGC***CTATGTGCCCTCAAGAGGGAGAT

[a] All primers are designed using the VP1 gene sequence of EV-71 MAS01/AH/CHN/09 (GenBank accession No. JQ409495). The number in the name of primer denotes the location of amino acid from N- or C-terminus of VP1, as F (forward) and R (reverse), respectively.

[b] Letters in boldface and italics denote the recognition sequences of restriction enzymes.

Table 2

Truncated proteins located at the N-terminus 100 aa of EV-71 VP1 and their WB reactivity with rabbit sera against EV-71, CV-A16 and E-6.

EV-71 fusion protein	Length (aa)	Predicted size (kDa)	WB reactivity with rabbit sera		
			anti-EV-71	anti-CV-A16	anti-E-6
GST-VP1$_{1–100}$	100	36.34	+++	++	+
GST-VP1$_{6–100}$	95	35.84	+++	+	+
GST-VP1$_{1–100}$	90	35.30	+++	+	+
GST-VP1$_{21–100}$	80	34.31	+	+	+
GST-VP1$_{1–50}$	50	31.10	+++	+	−
GST-VP1$_{1–40}$	40	30.16	+++	+	−
GST-VP1$_{1–30}$	30	29.02	+++	+	−
GST-VP1$_{6–50}$	45	30.60	+++	−	−
GST-VP1$_{6–43}$	38	29.95	+++	−	−

Table 3

Summary of blastp results using the EV-71 $VP1_{6–43}$ aa sequence as query against other enteroviruses.

Species	Serotypes	Identity (%)
A	Enterovirus 71	89.5–100
	Coxsackievirus A16	44–55
	Coxsackievirus A7	39–45
	Enterovirus 89–91	38–44
	Coxsackievirus A14	42
	Coxsackievirus A5	39–42
	Coxsackievirus A10	37–42
	Coxsackievirus A8	41
	Coxsackievirus A12	39
	Coxsackievirus A3	37
	Coxsackievirus A2	34–37
	Coxsackievirus A4	31–37
	Coxsackievirus A6	26–37
	Enterovirus 76	26–33
B	Coxsackievirus A9	–
	Coxsackievirus B1–6	–
	Echovirus 1–7, 9,11–21, 24–27, 29–33	–
	Enterovirus 69, 73–75, 77–78	–
C	Coxsackievirus A1, A11, A13, A15	–
	Poliovirus 1–3	23–29
D	Enterovirus 68, 70	–

"–" denotes no significant similarity found.

Table 4

Detection of anti-EV-71 IgM in acute-phase sera from 51 HFMD patients using GST-$VP_{16–43}$.

No.	Age (years)	Clinical diagnosis	RT-PCR[a]	NT[b]	Anti-EV-71 IgM	
					ELISA (OD value)[c]	WB
1	1	HFMD/aseptic meningitis	EV-71(+)	+	+ (0.524)	++
2	4	HFMD/aseptic meningitis	EV-71(+)	+	− (0.156)	+
3	1	HFMD/aseptic meningitis	EV-71(+)	+	+ (1.178)	++
4	1	HFMD/aseptic meningitis	EV-71(+)	+	+ (0.908)	++
5	4	HFMD/aseptic meningitis	EV-71(+)	+	− (0.123)	+
6	4	HFMD/aseptic meningitis	EV-71(+)	+	− (0.074)	−
7	1	HFMD/aseptic meningitis	EV-71(+)	+	+ (0.222)	+
8	2	HFMD/aseptic meningitis	EV-71(+)	+	− (0.106)	+
9	1	HFMD/aseptic meningitis	EV-71(+)	+	+ (0.405)	+
10	1	HFMD/aseptic meningitis	EV-71(+)	+	+ (0.932)	++
11	1	HFMD/aseptic meningitis	EV-71(+)	+	+ (0.443)	+
12	3	HFMD/aseptic meningitis	EV-71(+)	+	+ (0.857)	++
13	4	HFMD/aseptic meningitis	EV-71(+)	+	+ (0.223)	+
14	1	HFMD/aseptic meningitis	EV-71(+)	+	+ (1.414)	++
15	1	HFMD/aseptic meningitis	EV-71(+)	+	− (0.135)	++
16	1	HFMD/aseptic meningitis	EV-71(+)	+	+ (0.877)	++
17	4	HFMD/aseptic meningitis	EV-71(+)	+	+ (0.291)	++
18	3	HFMD/aseptic meningitis	EV-71(+)	+	+ (0.689)	++
19	1	HFMD/aseptic meningitis	EV-71(+)	+	+ (0.235)	++
20	2	HFMD/aseptic meningitis	EV-71(+)	+	+ (0.409)	++
21	2	HFMD/Myelitis	EV-71(+)	+	+ (0.233)	++
22	3	HFMD/aseptic meningitis	EV-71(+)	+	+ (0.483)	++
23	1	HFMD/aseptic meningitis	EV-71(+)	+	+ (0.411)	+
24	2	HFMD/aseptic meningitis	EV-71(+)	+	+ (0.559)	+
25	2	HFMD/aseptic meningitis	EV-71(+)	+	+ (0.294)	+
26	3	HFMD/aseptic meningitis	EV-71(+)	+	+ (0.665)	+
27	4	HFMD/aseptic meningitis	EV-71(+)	+	+ (0.462)	+

No.	Age (years)	Clinical diagnosis	RT-PCR[a]	NT[b]	Anti-EV-71 IgM	
					ELISA (OD value)[c]	WB
28	1	HFMD/aseptic meningitis	EV-71(+)	+	−(0.111)	−
29	2	HFMD/aseptic meningitis	EV-71(+)	+	− (0.097)	−
30	2	HFMD/aseptic meningitis	EV-71(+)	+	+ (0.346)	+
31	1	HFMD/aseptic meningitis	EV-71(+)	+	+ (1.042)	++
32	1	HFMD/aseptic meningitis	EV-71(+)	+	+ (0.749)	++
33	2	HFMD/aseptic meningitis	EV-71(+)	+	− (0.115)	+
34	2	HFMD/aseptic meningitis	EV-71(+)	+	+ (0.252)	+
35	2	HFMD/aseptic meningitis	EV-71(+)	+	+ (0.411)	+
36	1	HFMD/aseptic meningitis	EV-71(+)	+	+ (0.378)	+
37	2	HFMD/aseptic meningitis	CV-A16(+)	−	− (0.096)	−
38	2	HFMD	CV-A16(+)	−	− (0.086)	−
39	2	HFMD/aseptic meningitis	CV-A16(+)	−	− (0.143)	−
40	1	HFMD/aseptic meningitis	CV-A16(+)	−	− (0.019)	−
41	4	HFMD/aseptic meningitis	CV-A16(+)	−	− (0.107)	−
42	2	HFMD/aseptic meningitis	CV-A16(+)	−	− (0.056)	−
43	3	HFMD/aseptic meningitis	CV-A16(+)	−	− (0.061)	−
44	1	HFMD/aseptic meningitis	CV-A16(+)	−	− (0.057)	−
45	4	HFMD/aseptic meningitis	PEV(+)	−	− (0.087)	−
46	2	HFMD/aseptic meningitis	PEV(+)	−	− (0.089)	−
47	1	HFMD	PEV(+)	−	− (0.066)	−
48	5	HFMD/aseptic meningitis	PEV(+)	−	− (0.115)	−
49	2	HFMD	pev(−)	−	− (0.002)	−
50	2	HFMD/aseptic meningitis	pev(−)	−	− (0.072)	−
51	4	HFMD/aseptic meningitis	PEv(−)	−	− (0.039)	−

[a] EV-71(+): PEV(+)EV-71(+)CV-A16(−); CV-A16(+): PEV(+)EV-71(−)CV-A16(+); PEV(+): PEV(+)EV-71(−)CV-A16(−); PEV(−): PEV(−)EV-71(−)CV-A16(−); PEV: pan-enterovirus.

[b] Anti-EV-71 neutralizing (NT) antibody, cut-off level for seropositivity was ≥1:8.

[c] IgM results were judged using the cut-off OD value (=0.180).